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Original Article

Design, Codon Optimization, and Bioinformatic-Assisted Development of a Chimeric Circumsporozoite Protein for Malaria Vaccine Production Using Duckweed (*Lemna minor*) as a Plant Bioreactor

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HIGHLIGHTS

- Duckweed was utilized as a platform for recombinant protein production.
- A new chimeric circumsporozoite protein (CS712) from Malaria was optimized for expression in *Lemna minor*.
- The designed CS712 showed strong antigenicity and no allergenicity in bioinformatic analyses.
- Predicted epitopes highlighted robust B- and T-cell immune response potential.
- CS712 effectively interacted with TLR2, and could activate innate and adaptive immunity.

ABSTRACT

Malaria remains a serious global health problem, causing high morbidity and mortality, especially in tropical and subtropical regions. The development of effective and scalable vaccines is crucial for the control of this disease. This study investigates the application of *Lemna minor* (duckweed) as a novel, cost-effective, and sustainable bioreactor for the production of a chimeric circumsporozoite protein (CSP), a key antigen for malaria vaccine development. The synthetic CSP (CS712) was designed using duckweed-specific codon tables and codon-optimized to increase expression efficiency. Comprehensive bioinformatic analyses were performed to evaluate its physicochemical properties, antigenicity, allergenicity, and immunogenic potential. The optimized CS712 protein exhibited favorable properties, including high structural stability validated by Ramachandran plot and ProSA-Web, a PI of 5.31, and strong antigenicity (78.67%) without any allergenicity. Epitope predictions showed robust B and T cell responses, while molecular docking studies confirmed effective interaction with Toll-like receptor 2 (TLR2), highlighting its ability to activate innate and adaptive immunity. The results underline the potential of duckweed as a versatile plant platform for the production of recombinant vaccines. The CS712 protein proves to be a promising candidate for the development of a malaria vaccine and offers an innovative approach combining bioinformatic-based design and plant biotechnology. This study not only advances malaria vaccination strategies but also highlights the broader applicability of plant-based systems to address global health challenges through sustainable and scalable solutions.

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Introduction

Malaria is one of the most important parasitic diseases and a major health problem in tropical and subtropical countries, with a significant economic and social impact on the world (Kogan and Kogan, 2020). In 2019, an estimated 229 million cases of malaria were reported worldwide, including about 215 million in Africa, and about 409,000 deaths directly from the disease (WHO, 2022). The disease is caused by Plasmodium species, unicellular eukaryotic protozoa in the phylum Apicomplexa (Araujo et al., 2018). The five Plasmodium species that cause malaria in humans including *Plasmodium falciparum, P. vivax, P. malariae, P. ovale, and P. Knowlesi* (Antony and Parija, 2016).

The symptoms of malaria, including headache, aching limbs, chills, nausea, vomiting, and fever, usually appear 10-15 days after infection. Severe cases can lead to multi-organ problems in adults respiratory symptoms and anemia in children (Milner, 2018). Despite progress in containing malaria worldwide, it remains a major challenge in endemic regions, underscoring the need for continued efforts to eradicate the disease (Huang et al., 2022). Adults in endemic regions develop partial immunity, which prevents symptoms but does not eliminate the parasite, making them reservoirs for parasite transmission (Crompton et al., 2014; Midekisa et al., 2015). Major obstacles to malaria control include resistance to antimalarial drugs and insecticides, underscoring the urgent need for new intervention strategies, including vaccines, to mitigate the impact of malaria (Ippolito et al., 2021).

Recent advances in oral vaccine technology offer promising opportunities for vaccine development. Oral vaccines are inexpensive, needle-free, generally do not require a cold chain, and are painless to administer (Kwong et al., 2023). Unlike injectable vaccines, which are administered via the bloodstream, oral vaccines target mucosal surfaces and stimulate immune responses both locally and systemically (Jazayeri et al., 2021). The mucosal immune system is the primary defense against pathogens that invade via mucosal surfaces, making this approach ideal for the management of infectious diseases (Kim et al., 2016). The Malaria Vaccine Technology Roadmap aims to develop new malaria vaccines by 2030, which provide at least 75% protection against clinical malaria (Moorthy et al., 2013). Vaccine development focuses on different stages of the Plasmodium life cycle: pre-erythrocytic vaccines for sporozoites (Duffy et al., 2012), erythrocytic for asexual stages (Richards and Beeson, 2009), and transmission-blocking vaccines for sexual stages (Sagara et al., 2018).

Recent advances have improved our understanding of the development of vaccines, particularly subunit-based vaccines. (Tripp, 2021) These methods utilize computational tools to efficiently identify and screen vaccine candidates and immunogenic epitopes (Skwarczynski et al., 2020). The circumsporozoite protein (CSP), located on the surface of the plasmodium sporozoites, acts as an essential molecule for entry into host hepatocytes (Birkett, 2016). This molecule could play a critical role in malaria vaccine trials due to its role in triggering protective immunity (Duffy and Gorres, 2020). CSP could efficiently target antibodies and T cells that eliminate the pre-erythrocytic stages of the parasite (Cohen et al., 2010).

The CSP of Plasmodium vivax comprises three main domains: a central repeat domain and conserved N- and C-terminal regions. Antibodies targeting these domains have shown a protective effect in animal models (Yadava et al., 2014). Sequencing of the CSP genes identified three alleles, VK210, VK247, and P. vivax-like, which differ mainly in the central repeat domain (Gimenez et al., 2017). The malaria vaccine RTS, S/AS01, the first parasite vaccine for humans in Phase III trials, is based on the circumsporozoite surface protein (CSP) antigen. It has modest efficacy, and a short shelf life and requires a four-dose regimen for maximum efficacy (Stanisic and Good, 2023). Several recombinant malaria vaccines based on the CSP protein are currently in clinical trials; including EBA-175 RII NG, AMA-1 DiCo, MSP3 LSP and serine repeat antigen-5 (Tsoumani et al., 2023).

In the past decade, the newly aquatic plant expression systems (duckweed, Lemnaceae) were increasingly used for the production of recombinant proteins due to their cost efficiency and scalability (Tusé et al., 2014). Duckweeds are fast-growing aquatic monocotyledons found in stagnant or slow-moving freshwater environments such as ponds, lakes, and marshes (Thingujam et al., 2024). These tiny, 1-10 mm plants are composed of floating fronds and delicate roots (Feng et al., 2017). The unique characteristics of duckweeds, such as tolerance to low light, fluctuating nutrient levels, and different pH values, make them particularly suitable for artificial cultivation (Baek et al., 2021). The duckweed family has great diversity with over 30 species in five genera (*Lemna, Landoltia, Spirodela, Wolffia, and Wolfiella*) (Smith et al., 2024). Recent advances in multi-omics and gene transformation technology have enabled the successful expression of commercially important proteins, including vaccines, enzymes, and medicinal proteins, in duckweed species such *as L. minor, W. globosa, W. arrhiza, and S. oligorrhiza*. In 2001, Biolex Therapeutics demonstrated

that duckweed is an effective platform for the expression of recombinant proteins such as human growth hormone, monoclonal antibodies, and interferon-α2 (Gasdaska et al., 2003). Among the duckweed species, *Lemna minor* (*L. minor*) is most frequently used for the expression of exogenous proteins. Successful examples are the protective antigen of the porcine epidemic diarrhea virus (PEDV), the *M2e* gene of the avian influenza virus (H5N1), and the *HA* gene, which plays a crucial role in the production of the hemagglutinin protein for the H5N1 virus (Ko et al., 2011; Bertran et al., 2015; Firsov et al., 2015). *L. minor* has also demonstrated its effectiveness as a bioreactor for the production of industrial enzymes, achieving high yields of enzymes such as endoglucanase E1, β-glucuronidase, and hirudin (Sun et al., 2007; Kozlov et al., 2019). In addition, duckweed is used as a valuable source of protein in human and animal nutrition, including direct consumption of recombinant proteins such as vaccines (Heenatigala and Hou, 2024). The high growth rate, low risk of contamination, cost-effective cultivation, and scalability of duckweed make it an ideal plant for recombinant protein production (Khvatkov et al., 2021).

To further explore the potential of duckweed as an expression system for recombinant proteins, this study aims to generate a recombinant chimeric antigen with immunogenicity comparable to its native form. Specifically, two synthetic antigenic constructs of *P. vivax* CSP were designed based on the most common types, VK210 and VK247. These constructs, named CS127 and CS712, contain the primary sequences of the N- and C-terminal regions together with a fused central repeat region of VK210/VK247 and VK247/VK210 (Shabani et al., 2017). In this study, the CS712 construct was optimized for expression in duckweed by altering the nucleotide sequences based on the preference of duckweed codons. Comprehensive bioinformatic analyses were performed to assess RNA stability, physicochemical properties, secondary and tertiary protein structures, and to predict B and T cell epitopes. The optimized CS712 construct was then introduced into duckweed using a suitable vector, setting the stage for further experimental validation of the effectiveness of duckweed as a bioreactor for this antigen.

Materials and Methods

Chimeric CSPVK210/VK247 proteins

The sequences for *CS712* (accession No. KY548404) and *PvCSP* artificial genes (*VK210*, Sal-1 accession No. GU39059 and *VK247*, Papua New Guinea, accession No. M69059) were retrieved from the gene bank (www.ncbi.nlm.nih.gov/nuccore/KY548404.1). The N-terminal consists of 76 conserved amino acids preserved from the Sal-1 sequence, followed by 7 repeats of the VK247 motif (ANGAG [N/D] QPG), with four and three repeats of ANGAGNQPG and ANGAGDQPG, respectively. After these repetitions, the C-terminal region contains 69 amino acids, the beginning of which harbors a genetic insertion (GGNAANKKAEDA) in a 12-amino acid sequence. This sequence variant was derived from isolated data obtained from South Korea and Iran (Belem sequence, accession no. EU401923). At the end of this C-terminal region, a hexa-histidine (6 × His) tag is added to facilitate recombinant protein purification by nickel affinity chromatography (**Fig. 1**).

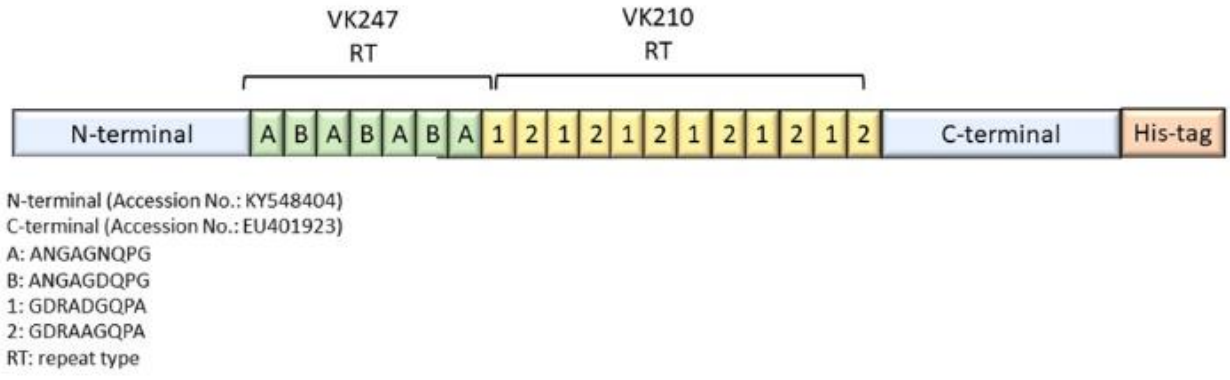

Figure 1: Structure of the CS712 protein, a chimeric protein developed for a malaria vaccine. This protein consists of the VK210 and VK247 sequences of *Plasmodium vivax* and the N- and C-terminal regions. CS712 contains 7 repeats of the VK247 motif (ANGAG [N/D] QPG) and 12 repeats of the VK210 motif (GDRA [D/A] GQPA). The C-terminal region contains a genetic insertion from South Korean and Iranian isolates. A hexa-histidine tag is added to the C-terminal end.

Optimizing the structure for expression in Duckweed

As part of the *CS712* sequence optimization, a plant-specific Kozak sequence and initial GC nucleotides were added to enhance translation efficiency (Kozak, 1989). To prevent further glycosylation and retain the protein within the endoplasmic reticulum, the SEKDEL sequence was incorporated at the C-terminal of the synthetic construct (Denecke et al., 1992). The GenScript software and the Duckweed Codon Table were used to optimize the CS712 coding sequence for efficient expression in *L. minor*. The aim of the optimization was to improve the compatibility of codon usage with the host organism by increasing the Codon Adaptation Index (CAI) and adjusting the GC content for stable mRNA translation. In addition, rare codons that could reduce translation efficiency were replaced with synonymous codons favored by duckweed. Online RNAfold software was employed for RNA structure prediction and analysis.

Analysis of physicochemical, antigenicity, and allergenicity parameters

To predict the physico-chemical parameters of the artificial protein CS712, the ExPASy ProtParam software (web.expasy.org/protparam) was used. The molecular weight, the molar extinction coefficient, the isoelectric point (pI), the number of positive and negative residues, the half-life of the oligomeric protein, the aliphatic index as a factor for improving the heat resistance of spherical proteins (Ikai, 1980), GRAVY (grand average hydropathy) (Kyte and Doolittle, 1982), and instability index (Gasteiger et al., 2005) were calculated. In addition, the antigenicity of the vaccine was predicted with VaxiJen v2.0 and ANTIGENpro. ANTIGENpro (http://scratch.proteomics.ics.uci.edu/) estimates protein antigenicity based on data from protein microarray analysis (Sanami et al., 2022), while allergenicity was assessed using AlgPred, SDAP, ADFS, AllerTop, and Allergome.

Prediction of secondary and tertiary structures

GOR IV (Garnier-Osguthorpe-Robson) secondary structure prediction online service was used for protein secondary structure forecasting (Garnier et al., 1996). Additionally, the I-TASSER server is used to calculate the confidence score (C-score) using the tertiary or three-dimensional (3D) model (Zhang, 2008). In I-TASSER, similar structure patterns can be identified from the Protein Data Bank (PDB) based on the sequence-to-structure-to-function paradigm. The highest confidence score indicates the more suitable model (Yang et al., 2015). PyMOL software was used to observe the protein structure in 3D (Lill and Danielson, 2011).

Optimizing energy and assessing protein structure prediction credibility

The ProSA-web server (https://prosa.services.came.sbg.ac.at/prosa.php) estimates the quality score of a protein 3D structure, which is represented by the Z score (Wiederstein and Sippl, 2007). In addition to Ramachandran plots, RAMPAGE was used to assess the quality of the structural optimization (Laskowski et al., 1993; Lovell et al., 2003), The C-score from the I-TASSER server was used to estimate the quality of the predicted model.

Epitope prediction for B-cells

Based on physicochemical properties, the linear B-cell epitopes were predicted using BcePreds and AAPreds (EL-Manzalawy et al., 2008). Physicochemical criteria, such as hydrophilicity, flexibility, mobility, accessibility, polarity, surface exposure, and antigenicity, are used to predict linear B-cell epitopes on the BcePred server. The authentic protein model was analyzed for discontinuous epitopes of B cells using ElliPro (http://tools.iedb.org/ellipro/) (Jaiswal et al., 2020) T-cell epitope prediction was carried out to identify potential T-cell epitopes. Additionally, interferon-gamma (IFN-γ) inducing epitopes were predicted using the IFNepitope server to assess their effectiveness in stimulating cellular immunity (Wang et al., 2010; Dhanda et al., 2013).

Exploring the dynamics of the protein-TLR2 receptor interaction.

To validate the interaction between the recombinant protein and Toll-like receptor 2 (TLR2), SwarmDock software (http://bmm.crick.ac.uk/~SwarmDock/index.html) was used, which applies a flexible protein-protein complex model. The process begins with the minimization and preprocessing of input structures, including repairing bonds, modeling missing atoms, and applying CHARMM force fields. Docking is performed using a combination of particle swarm optimization and local search algorithms. Finally, the resulting docked poses are minimized, re-ranked, and clustered to determine the most accurate interactions.

Results and Discussion

Optimizing chimeric gene

Kozak (G/ACCACCATGGC) sequence was inserted at the translation start site (ATG) to accurate and improve the protein expression level. Three essential nucleotides are present in the Kozak sequence for plant hosts at positions A (-3), G (+4), and C (+5). The GCT codon

(Ala) sequence was added downstream of the ATG codon to include these nucleotides, which were not present in the current study gene sequence. Since plants use the UGA stop codon more frequently, it was added to the end of the sequence (Angenon et al., 1990). Synthetic chimera was analyzed for codon bias and GC content using the amino acids of the CS712 construct back-translated to nucleotide sequence using duckweed codon usage. Additionally, the Codon Adaptation Index (CAI) was applied to assess the suitability of the synthetic construct for high-level expression in *L. minor*. This index compares the relative adaptation of codon usage in the CS712 gene to highly expressed genes in duckweed. The CAI value was found to be 0.86, confirming effective translation optimization. The minimum energy content of the mRNA structure was found to be -422 kcal/mole. This indicates that the mRNA adopts a conformation with the lowest possible energy, signifying its most stable structural state for efficient translation.

Duckweed has emerged as a bioreactor for protein biosynthesis, offering high protein yields and stable storage of target proteins. In addition to vaccine production, duckweed is also suitable for the expression of antibodies, pharmaceutical proteins, and industrial enzymes, making it a versatile platform for various biosynthetic applications (Yang et al., 2021). In this study, we utilized bioinformatic tools to optimize a chimeric and multiepitopic gene for expression in the duckweed species *Lemna minor*, demonstrating its potential for the production of valuable biologics.
Bioinformatics has become an important tool for the advancement of biological research, especially for the identification of protein antigens for vaccine development. In silico approaches provide valuable insights into the stability, energy profiles, and functionality of proteins and greatly accelerate the transition from conception to preclinical testing (Oli et al., 2020). These computational methods offer time and cost savings by enabling the design and evaluation of multiple antigen structures through simulations. Immunoinformatics also helps to select the most promising immunogenic agents for chimeric multi-subunit vaccines, increasing the efficiency and effectiveness of vaccine development processes (Kupani et al., 2023).

Optimizing codon usage can significantly enhance the efficiency of recombinant protein production. For instance, codon optimization increased the expression of stem cell factor (SCF by 25- to 30-fold in tobacco Bright Yellow-2 (BY-2) cells, illustrating its impact on boosting yield (Kulshreshtha et al., 2022). In the case of the CS712 construct, codon sequences were tailored for duckweed, and mRNA predictions confirmed effective translation. In this study, codon optimization was tailored to duckweed and resulted in a CAI of 0.86, which is in the optimal range for efficient translation. The minimal free energy of the predicted mRNA structure (-422 kcal/mol) supports its stability and enables improved expression. These modifications are consistent with previous findings in which codon alignment significantly increased the production of recombinant proteins in plant systems. Additionally, optimizing the 5'-untranslated regions of mRNAs played a crucial role in enhancing translation rates and improving transcript stability (Chu et al., 2024).

Physicochemical characteristics

The physicochemical characteristics of the Lemna-optimized synthetic and the reference (CS712, accession no. KY548404) constructs are summarized in Table 1.

Table 1. A comparison of the physicochemical characteristics of references CS712 (Acc. No. KY548404) and the chimeric structure developed in this work.

Sequences	Cs712ref	Cs712
Negatively charged residues (Asp + Glu)	46	49
Positively charged residues (Arg + Lys)	37	38
Number of aa[1]	358	365
Mw[2] (kda)	35.013	35.786
Pi[3]	5.49	5.31
Extinction coefficient (with/ without cys)[4]	7240/6990	7240/6990
Half-life[5]	30 h, > 20h, > 10h	30 h, > 20h, > 10h
Instability index[6]	24.46	23.24
Aliphatic index	39.58	40.16
Grand average of hydropathicity	−1.132	-1.137

The reference sequence for this study is CS712 ref (Acc No. KY548404), according to Shabani et al 2017.
[1] aa: amino acid.
[2] MW: molecular weight.
[3] pI: isoelectric point.
[4] Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.
[5] mammalian reticulocytes, yeast, E. coli (All in vivo).
[6] A protein whose instability index is smaller than 40 is predicted as stable.

Physicochemical analysis performed with ProtParam software revealed that the chimeric CS712 protein exhibits acidic properties, evidenced by a pI value of 5.31, similar to that of the natural CSP gene (pI = 5.31). The incorporation of alanine into the N-terminal region has been used as a strategy to optimize translation efficiency by improving ribosomal binding. This method has already been shown to improve protein expression

(Shang et al., 2023). Importantly, the incorporation of alanine did not alter the acidic properties of the construct, as its neutral nature facilitated the retention of these properties. The predicted high extinction coefficient at 280 nm further validated this observation and was corroborated by the significant protein expression yield achieved. Furthermore, the stability of the recombinant CS712 chimeric structure was affirmed by ExPASy ProtParam, which evaluated its instability index, molecular weight, high aliphatic index, and low GRAVY score, indicating that the protein is stable, thermostable, hydrophilic, and a suitable antigen for recombinant chimeric proteins.

Assessment of antigenicity and allergenicity

The antigenicity of the recombinant protein was predicted to be 78.67% by ANTIGENpro and 65.72% by Vaxijen. The results indicated that the fusion protein and its components exhibited the highest probability of antigenicity index, which exceeded the threshold set by the software. This finding confirms that the selected regions possess strong antigenicity. Furthermore, allergen analysis of the protein fragment demonstrated that the designed fusion protein does not classify as an allergen. Therefore, immuno-informatics analyses confirmed that the fusion construct is a robust antigen and not an allergen, which is important data in chimeric antigen design.

Prediction of secondary and tertiary structures

According to GOR IV, the secondary structure of CS712 is composed of mostly random coils (82.74%) and alpha helices (12.33%) (Fig. 2). The I-TASSER server was successfully used to model the chimeric CS712 protein. Fig. 2 illustrates the predicted chimeric structure of CS712, comprising three distinct domains. The model quality was assessed using the C-score (0.3), which typically ranges from -5 to 2, with higher values indicating greater confidence in the model (Yang and Zhang, 2015).

Evaluation of model stability

The structural quality and reliability of the CS712 chimeric construct were evaluated using a Ramachandran plot (Lovell et al., 2003). The analysis revealed that 358 amino acid residues (98.6%) are in the favored region, 3 residues (0.8%) are in the allowed region, and only 2 residues (0.6%) fall within the outlier region (Fig. 3A). Additionally, the ProSA web server calculated a Z-score of −5.77 for the model, indicating a good-quality structure (Fig. 3B). The results of the Ramachandran plot and the ProSA server also indicated that the model quality was clearly adequate. Furthermore, the RNA secondary structure prediction using Mfold software suggests favorable stability and translation efficiency (data not shown).

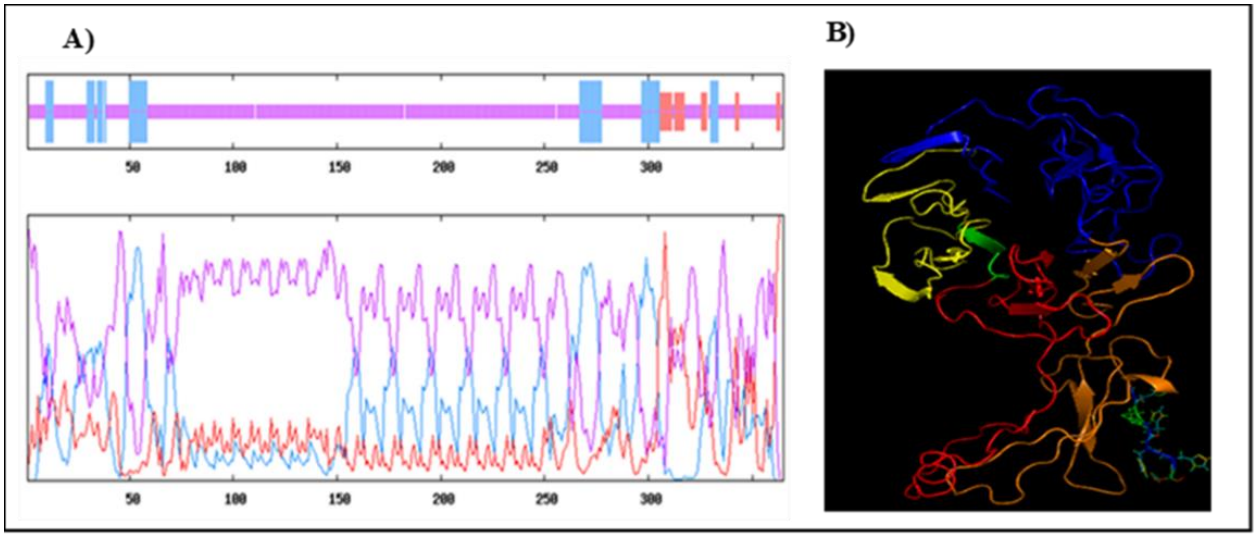

Figure 2: Predicted Secondary and Tertiary Structures of the CS712 Chimeric Construct. (A) Graphical representation of the predicted secondary structure of the CS712 chimeric construct protein. Helices, coils, and extended strands are shown in purple, red, and blue, respectively. (B) Predicted 3D structure of the CS712 protein construct, generated using I-TASSER software (Zhang 2008) and visualized with PyMOL v1.7.2 (Zhang 2008). The model features three main domains: the N-terminal (red), repeat regions (yellow and blue), and the C-terminal (orange). The green color represents the pre-repeat region of VK247 (EDGAGNQPG). The repeat types of VK210 and VK247 are shown in blue and yellow, respectively. The His6 tag is located at the C-terminus to purify the protein. These predicted structures help us to better understand the structure of CS712 and how it might interact with TLR2.

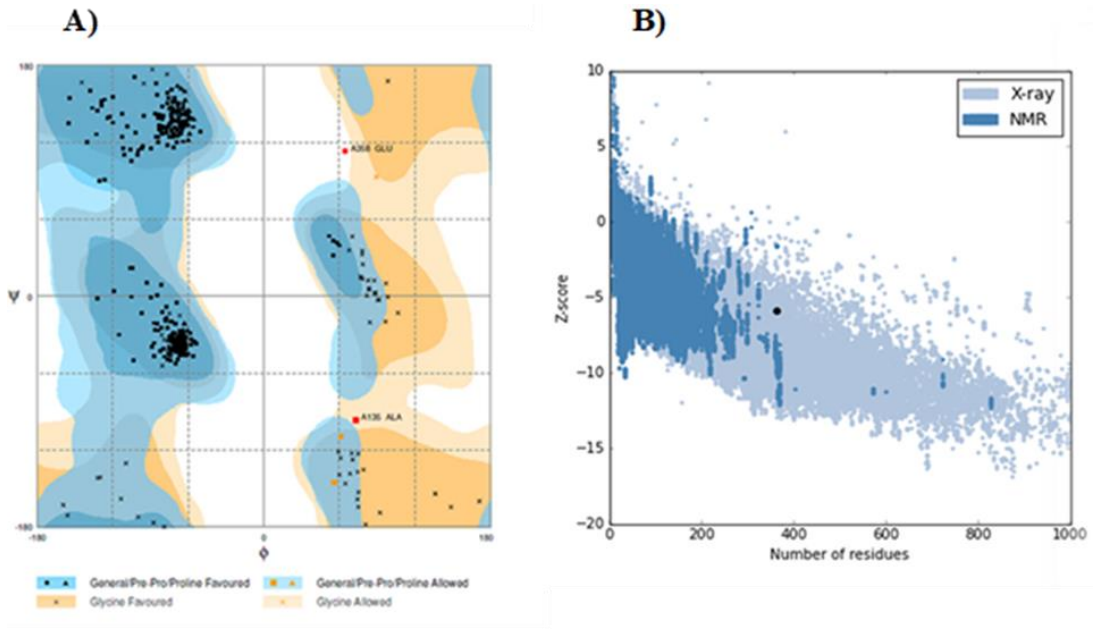

Figure 3: Ramachandran plot and ProSA-web validation of CS712 chimeric construct. (A) Ramachandran diagram: the distribution of dihedral angles shows a high quality of the predicted structure with 98.6% of the residues in the preferred region. (B) ProSA-web Z-score: -5.77, indicating high structural quality. These data indicate that the CS712 structure is of high quality and suitable for further studies.

Prediction of B-cell, T-cell, and IFN-gamma-inducing epitopes in CS712

Epitope prediction for the CS712 chimeric protein, reveals several high-affinity epitopes at the N- and C-termini (Table 2). Continuous B-cell epitopes were selected based on properties such as hydrophilicity and antigenicity (Table 3). Discontinuous B-cell epitopes, interferon-gamma (IFN-γ) inducing epitopes, and MHC class-II epitope regions were predicted and are summarized in Table 4. The most accurate epitope for the discontinuous B-cell epitope is highlighted in Fig. 4.

Table 2. BcePred-based linear B-cell epitopes within full-length proteins

Rank	Sequence	Start position	Score
1	HVGQSASRGRGLGENP	33	0.95
2	DGAGNQPGANGAGNQP	79	0.94
2	LGENPDDEEGDAKKKK	44	0.94
3	NGAGDQPGANGAGNQP	97	0.93
3	NGAGDQPGANGAGNQP	133	0.93
3	NGAGDQPGANGAGNQP	115	0.93
4	NGAGNQPGANGAGDQP	88	0.92
4	NGAGNQPGANGAGDQP	124	0.92
4	NGAGNQPGANGAGDQP	106	0.92
5	NGAGNQPGGDRADGQP	142	0.91
6	CGVGVRVRRRVNAANK	322	0.88

Table 3. Prediction of linear B-cell epitope regions of the CS712 chimeric protein at BcePred site

Prediction parameters	Threshold	Epitope
Hydrophilicity	1.9	22-28/35-32/333-341/348-359/
Flexibility	2	32-43/46-78/142-150/269-275/280-289/293-300/333-338/349-356
Accessibility	1.9	36-42/44-89/146-256/268-279/248-308/325-344/352-363
Exposed surface	2.3	46-79/271-278/295-307327-343
Polarity	1.8	40-79/271-279/295-308/324-343/352-365
Antigenic propensity	1.9	4-10/316-332/358-365

The development of an effective vaccine requires the incorporation of well-optimized epitopes for B and T cells to elicit a robust humoral and cellular immune response. The chimeric protein CS712 contains both continuous and discontinuous B-cell epitopes, enabling it to elicit a strong antibody response. The development of subunit vaccines also requires the selection of epitopes with high binding affinity (>1000) to MHC molecules and those that can stimulate interferon-gamma. Analysis by reputable servers indicates that the designed synthetic protein is likely to elicit effective humoral and cellular immune responses, highlighting its potential for vaccine development and similar applications.

Table 4. Predicted discontinuous B-cell epitope regions of the CS712 protein using the Ellipro site (Threshold: 0.5), IFN-gamma inducing epitopes identified by the IFNepitope server, and MHC class-II epitope regions predicted using the MHC 2pred site (Threshold: 0.5)

	Epitope	Score
discontinuous B-cell	VKEYLDKVRATVGTEWTPCSVTCG VGVRVRRRVNAANKKPEDLTLNDL ETDVCTSEKDELHHHHHH	0.79
	GAGNQPGANGAGNQPGANGAGDQ PGANGAGNQPGANGAGDQPGANG AGN	0.779
	AGQPAGDRADGQPAGDRA	0.696
	GVNFNNVDASSLGAAHVGQS	0.659
	RADGQPAGDR	0.589
	GQGQN	0.572
	AEPK	0.548
	AGDR	0.514
	GLGEN	0.507
IFN-gamma	KVRATVGTEW	10
	DLTLNDLETDVCTSEKDELH HHHHH	3
	ANGAGNQPGGDRADGQPAGDR	1.74
	GVRVRRRVNAANKKPEDLTL ND	1.42
	QPAGDRAAGQPAG	1.28
	DQPGANGAGNQPGGDRA	1.1
	KYQLTLFPHQFINPR	1
	GQSASRGRGLGENPDDE	0.74
MHC class-II	NLNGVNFNNVDASSLGA	
	HCGHNVDLSKAINLN	
	AHCGHNVDLSKAINLNG	
	CGHNVDLSKAINLNGVN	
	GHNVDLSKAINLNGVN	
	VNFNNVDASSLGAA	
	LNGVNFNNVDASSLG	
	HCGHNVDLSKAINLN	
	AHCGHNVDLSKAINLNG	

Investigating the interaction dynamics between protein and TLR2 receptor

Protein-protein docking between CS712 and TLR-2 was performed using SwarmDock software (Figure 5). The highest scoring model (Model 1) was selected based on its overall docking score of -887.8, electrostatic energy of -432.1, and van der Waals energy of -455.7, as well as its biologically plausible structure. This model reflects the most favorable interactions between the proteins. The binding interface comprises key residues of CS712 (Lys123, Arg125, Glu127) and TLR-2 (Asp234, Tyr236, Arg238) stabilized by hydrogen bonds, salt bridges and hydrophobic interactions. The distance between the centers of mass at the binding interface is 5.6 angstroms, and the contact area is 1250 square angstroms. The observed electrostatic and van der Waals interactions at the binding interface, including hydrogen bonding between key CS712 and TLR2 residues, are consistent with the interaction patterns reported for TLR2 ligands. This agreement supports the reliability of the computational predictions and suggests that CS712 can potentially influence immune signaling pathways via TLR2.

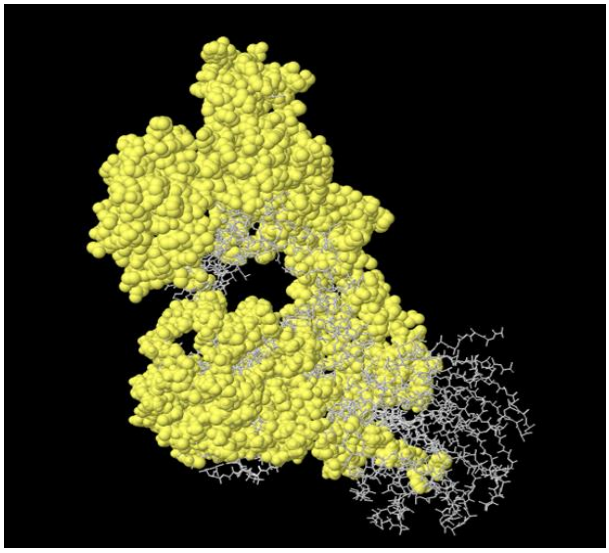

Figure 4: 3D demonstration of CS712 chimeric proteins showing conformational or discontinuous B-cell epitopes. There are yellow surfaces for conformational or discontinuous B cell epitopes, and grey sticks for the other residues (scoring 0.79). This epitope is suitable for subunit vaccine design.

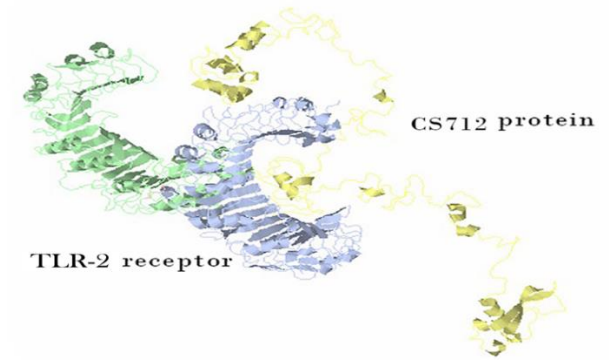

Figure 5. CS712 Protein-TLR2 Receptor Interaction Analysis. Shown is the docking model created with SwarmDock (Model 1), which scored the highest. The binding interface includes key residues of CS712 (Lys123, Arg125, Glu127) and TLR-2 (Asp234, Tyr236, Arg238), which are stabilized by hydrogen bonds, salt bridges and hydrophobic interactions. A total of four hydrogen bonds with bond lengths of 2.9, 3.1, 2.8, and 3.0 Angstroms are observed. The distance between the centers of mass at the bond interface is 5.6 Angstroms, and the contact area is 1250 square Angstroms. The docking model was selected based on its overall score (-887.8), electrostatic energy (-432.1), and van der Waals energy (-455.7), emphasizing its biological relevance and stability.

Toll-like receptors (TLRs) play a critical role in the innate immune response to malaria by recognizing invading *P. falciparum* sporozoites and initiating a signaling cascade that leads to the production of essential pro-inflammatory cytokines such as interferon-gamma (IFN-γ), interleukin-12 (IL-12), and tumor necrosis factor-alpha (TNF-α), which are essential for the clearance of the infectious agent (Kalita et al., 2023). The correspondence analysis between the CS712 protein and the TLR2 receptor revealed that the protein is predominantly hydrophilic, with specific hydrophobic regions that are critical for its interaction with the TLR2 receptor (Kang et al., 2009). Toll-like receptors (TLRs), which have been extensively studied and are found on the surface of antigen-presenting cells (APCs), may play a crucial role in this interaction (Vu et al., 2017). When vaccine components interact with these receptors, this ensures that the vaccine antigen is recognized by the APC-specific receptors. This recognition triggers innate immune responses and subsequently activates the adaptive immune response. In this study, the hydrophobic regions of the CS712 protein and its associated immunodominant epitopes interact with TLR2, highlighting its important role in triggering the immune response.

The results of molecular docking showed that CS712 can potentially interact with TLR2. The observed electrostatic and van der Waals interactions at the binding interface, including hydrogen bonds between key residues of CS712 (Lys123, Arg125, Glu127) and TLR2 residues (Asp234, Tyr236, Arg238), are consistent with the interaction patterns reported for TLR2 ligands. This agreement supports the reliability of the computational predictions and suggests that CS712 may influence immune signaling pathways via TLR2 (van Bergenhenegouwen et al., 2013). Compared to other TLRs, TLR2 is unique in that it must form heterodimers with TLR1 or TLR6 to initiate signaling and cellular activation (Li et al., 2014). This property could influence the type of immune response triggered by CS712. In addition, the use of TLR2 adjuvants could enhance the immune response to CS712 and increase the efficacy of the vaccine. CS712 has considerable potential as a malaria vaccine antigen. Its high immunogenicity, structural stability and potential to induce protective immunity make it an attractive candidate for vaccine development. In addition, CS712 could be integrated into multivalent vaccines to provide protective immunity against multiple stages of the Plasmodium parasite life cycle. Future research could focus on investigating the detailed mechanism of CS712 interaction with TLR2, evaluating the efficacy of TLR2 adjuvants in the CS712-based vaccine, and exploring the potential of CS712 in multivalent vaccines.

Importance and limitation of this study

Malaria is a life-threatening disease that causes significant morbidity and socioeconomic burden, exacerbated by drug-resistant parasites and insecticide-resistant mosquitoes. While the effectiveness of vaccination in controlling infectious diseases is well established, traditional vaccine production methods often face significant safety concerns and manufacturing challenges. These issues are particularly pronounced for malaria, underscoring the urgent need for more efficient and scalable vaccine manufacturing strategies (Skwarczynski et al., 2020).

Recombinant subunit vaccines offer a promising advance by enabling precise antigen design and incorporating cutting-edge technologies such as nanotechnology. These innovations eliminate many of the limitations of traditional vaccines and increase both specificity and safety (Cid and Bolívar, 2021). A key crucial element in this approach is the circumsporozoite protein (CSP), essential for the initial invasion of the malaria parasite into the host hepatocytes and therefore represents a primary target for malaria vaccines. The chimeric protein CS712, derived from the repeat regions of the PvCSP, has emerged as a promising vaccine candidate. Previous studies show that CSP achieves satisfactory levels of expression, purity, and specific antibody recognition, highlighting its potential as an effective vaccine against Plasmodium vivax (Esquenazi, 2023). Despite decades of research, no licensed malaria vaccine is available due to challenges such as the pathogen's immune escape through antigenic variation and human leukocyte antigen (HLA) diversity. These factors complicate vaccine development by hindering the immune system's ability to recognize and respond effectively to the parasite. Designing vaccines that address this variability is crucial to achieving broader protection (Pritam et al., 2020). Variant surface antigens (VSAs), including PfEMP1, RIFINs, SURFINs, and STEVOR families, are critical in this immune evasion process. The high variability in these antigens allows the parasite to continuously alter its surface epitopes, enabling it to evade recognition and destruction by the host's immune system, thereby ensuring its survival (Walker and Rogerson, 2023). The integration of such advanced recombinant subunit vaccines into malaria control strategies could significantly improve vaccine efficacy and fill critical gaps in existing technologies. In addition, the development and distribution of effective malaria vaccines are crucial not only to preventing severe illness and death but also for enhancing community health and enabling economic stability in regions heavily impacted by the disease (Adeyemo et al., 2022).

Genetically engineered plants are capable of producing therapeutic proteins and vaccines, significantly reducing reliance on costly bioreactors and specialized culture media. While challenges remain — such as the time required to develop transgenic plants and the complexity associated with achieving high yields of complicated proteins-plant-based systems represent a compelling alternative. These platforms are inherently versatile and adaptable, enabling rapid response to emerging health threats and effectively meeting the need for vaccines (Shanmugaraj et al., 2024). Plant Molecular Farming (PMF) stands out as a scalable and flexible approach for the production of recombinant proteins and biopharmaceuticals, making it a competitive option over traditional expression systems such as bacterial cultures, yeast systems, and mammalian cell lines. Numerous biopharmaceuticals, including recombinant vaccine antigens, monoclonal antibodies, and other commercially viable proteins, are produced in plants, some of which are currently in preclinical and clinical development (Shanmugaraj et al., 2020).

In this study, a novel vaccine candidate, CS712, was designed for malaria parasites using bioinformatic tools. Although the currently designed vaccine candidate, CS712, is a promising vaccine candidate based on bioinformatic predictions, it is important to note that these predictions have limitations. In silico models, such as protein-protein docking, should be validated by experimental studies as they may not fully represent real biological conditions. In addition, potential immunogenic risks, including adverse immune reactions, should be assessed. Further in vitro and in vivo testing is required to confirm the safety and efficacy of CS712 before conducting clinical trials. In addition, our proposed system faces challenges in production scalability, purification, stability, delivery, and immunogenicity of the duckweed-based vaccine. Other limitations of this system include the complexity of the chimeric vaccine design, the limitations of bioinformatic studies, and the need for additional experimental studies.

Conclusion

Vaccination is essential for combating infectious diseases, but traditional methods face significant safety and production problems, especially in malaria. Recombinant subunit vaccines, such as those based on the Plasmodium vivax circumsporozoite protein (CSP), offer advances in precise antigen design and improved safety. The chimeric protein CS712 is a promising candidate for an effective malaria vaccine due to its favorable expression and immunogenicity profile. Plant-based production systems, particularly using duckweed, offer a cost-effective and scalable alternative for vaccine manufacturing. Despite some challenges, these systems offer flexibility and high yields, making them suitable for rapid vaccine production. Bioinformatics tools have played a critical role in optimizing vaccine design by providing insights into protein stability and functionality. The successful optimization of codon usage for recombinant protein expression in duckweed supports the efficacy of the CS712 protein as a stable and effective antigen. Furthermore, the interaction of CS712 with TLR2 receptors underscores its potential to activate both innate and adaptive immune responses.

In summary, the integration of advanced recombinant subunit vaccines and innovative production systems represents a significant step forward in overcoming the challenges of vaccine development and improving malaria control strategies.

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Ethical Statement

The authors confirm that this study complies with institutional regulations and ethical guidelines and does not involve human or animal subjects. Furthermore, the manuscript does not violate copyright, third-party rights, or other applicable laws.

Competing Interests

The authors declare that there are no competing financial and non-financial interests, direct or indirect, related to this manuscript.

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Data Availability

Additional data will be available from the corresponding author upon reasonable request.

Authors Contribution

AHS was responsible for designing the project and leading the research. NR, a PhD student, and project manager, supervised and coordinated the research

activities. MA, ET, and FF were in charge of data analysis and organizing the research database. All authors read and approved the final manuscript.

Declaration of AI Tools Usage

We gratefully acknowledge the use of artificial intelligence (AI) tools solely for the purpose of linguistic revision and to improve the clarity of the manuscript. The content, analysis, and scientific integrity of the manuscript are the sole responsibility of the authors.

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